User Instructions

X-Lyz™ Conjugation Density Kit

For Quantitation of Conjugation Density

Catalog # IC286099

Features:

- ***15min quick assay***
- ***Compatibility with reducing agents***
- ***Wide linear range up to 6000nmol/mL***
- ***Suitable for microplates and cuvettes***

For safely handling the reagents, refer to the MSDS sheets online:
www.x-lyz.com

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1 Product Description

Conjugation with amino groups in proteins is commonly achieved by NHS ester or EDC-like agents. However, these reagents are quickly hydrolyzed in an aqueous media. For instance, the NHS ester may decompose to its parent inactive acid completely in less than 10min; its half time may drop by 10-30 fold as the pH is raised from 7.0 to 8.5. The final conjugation ratios could be very inconsistent especially in dilute solutions. It is a requirement to have a simple and reliable analytical tool to determine the end result out of a particular reaction and to ensure its reproducibility.

The X-Lyz™ Conjugation Density Assay Kit is designed to provide a quick quantitative analysis for conjugation ratios. It measures the amine contents in the protein before and after the reaction. The amount of conjugated amines and ratio may then be obtained. In practice, there is no need to know the actual protein concentration in the sample. The assay involves a reaction with primary amines to form a chromophore. It exhibits a strong UV absorbance at 350nm linearly proportional to concentrations over a wide range of 25-6,000nmol/mL. The rapid color development is complete after 15min incubation. The assay is easy to operate compared to other methods, such as trinitrobenzenesulfonic acid (TNBS), known as a dangerous explosive substance. The X-Lyz™ reagent is compatible with commonly used materials in the laboratory, such as reducing agents, detergents, alcohols, carbohydrates, and inorganic salts. The method is suitable for assays in microplates and test tube (cuvettes).

The X-Lyz™ Conjugation Density Kit, Catalog # IC286099, is sufficient for up to 3200 assays in microplates and 480 assays in test tubes. The contents are specified in the table below.

Table 1: Contents in X-Lyz™ Conjugation Density Kit (Cat No. IC286099)

<table>
<tr><th>Items</th><th>Cat No.</th><th>Size</th><th>Contents</th><th>Store</th><th>Ship</th></tr>
<tr><td>Reagent A</td><td>IC286091</td><td>200mL</td><td>Containing Reagent A in aqueous solution, pH ~10.5</td><td>Room Temp</td><td rowspan="2">Room Temp</td></tr>
<tr><td>Reagent B</td><td>IC286092</td><td>40mL</td><td>Containing Reagent B in organic mixture</td><td>4ºC</td></tr>
<tr><td>Instructions</td><td>Pub286100</td><td>N/A</td><td>Instructions for X-Lyz™ Conjugation Density Kit</td><td>N/A</td><td>N/A</td></tr>
</table>

All reagents expire one year from the date of purchase.

The assay procedure simply involves mixing the Working Reagent (WR, prepared by combining reagents A and B at a ratio of 5/1) with samples, incubating for 15min and taking UV measurement at 350nm.

2 Preparation of Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   Total Volume of WR Required = # of Samples x (Volume of WR per Replicate) x (# of Replicates) x 1.05 (5% extra)

2. Prepare WR by mixing five parts of Reagent A and one part of Reagent B. Use the WR as soon as possible; sitting too long may cause loss of activity.

3 Determination of Conjugation Density

The determination of conjugation density involves UV absorbance measurements of amine concentrations in the samples before and after conjugation reactions. By comparing the results, the conjugation density may be estimated.

The X-Lyz™ assay procedure is illustrated with the following example in the biotinylation of an antibody.

3.1 Preparation of Solutions

- Bicine Buffer: Add a proper amount of bicine in DI water and adjusted to pH 8.3 with dilute NaOH and/or HCl.
- Biotin NHS Ester Solution: Add a proper amount of biotin NHS ester in anhydrous DMSO

3.2 Conjugated, Unconjugated, and Control Samples

- Conjugated Sample: Add proper amounts of Biotin NHS Ester Solution and antibody to Bicine Buffer, incubate 1hr at room temperature.
- Unconjugated Sample: Add a proper amount of Biotin NHS Ester Solution to Bicine Buffer and incubate 1hr at room temperature. The NHS Ester should be completely hydrolyzed under the condition. Then add a proper amount of antibody to the hydrolyzed biotin-bicine buffer solution. The concentrations of antibody and biotin NHS ester should be the same as those in the Conjugated Sample, respectively.
- Biotin Control Sample: Add a proper amount of Biotin NHS Ester Solution to Bicine Buffer; the concentration of Biotin NHS Ester should be the same as that in the Conjugated Sample. Incubate 1hr at room temperature.

Note: The solutions and samples must be free of any primary amine-containing substances other than that in the protein, such as TRIS, free amino acids or ammonium ions. Non-primary amine buffers may be used instead, e.g. HEPES, BICINE, BIS-TRIS, BIS-TRIS propane, and BIS-TRIS methane.

3.3 Determination of Conjugation Density

WR/Sample ratio of 10/1 or 1/1 may be used. The 10/1 ratio gives a wider analytical range, while 1/1 is a more sensitive procedure. The assay in the procedure below is conducted with a microplate reader; a spectrophotometer may be used as well in a similar manner.

3.3.1 WR/Sample 10/1 in Microplate

The procedure has a linear range of 150-6000nmol/mL (primary amine concentration)

1. Pipette 25µL of each Conjugated, Unconjugated, and Biotin Control Sample into a well in three or more replicates.
   Note: Different sample sizes may be used as long as the WR/Sample is 10/1. If the sample size is small, e.g. 10µL, it is recommended to use half-area microplates to maintain the detection sensitivity.
2. Add 250µL WR into each well and shake gently. Cover the plate with parafilm.
3. Incubate for at least 15min at temperature between 25-37°C.
4. Remove parafilm and measure UV absorbance at 350nm in a plate reader.
5. Subtract the average absorbance of Biotin Control from that of Conjugated and Unconjugated sample replicates to obtain the corresponding corrected absorbance (CA).
6. Use the formula to calculate the Conjugation Ratio.

   Conjugation Ratio = (Unconjugated's CA - Conjugated's CA) ÷ Unconjugated's CA) x (# of Lys + # of N-Termini)

   Where
   Conjugation Ratio is the number of molecules (biotin molecules in the case) attached to one protein (antibody);
   Conjugated's CA is the corrected absorbance of Conjugated Sample;
   Unconjugated's CA is the corrected absorbance of Unconjugated Sample;
   # of Lys is the number of un-blocked lysine in the protein before conjugation;
   # of N-termini is the number of un-blocked N-termini in the protein before conjugation.

3.3.2 WR/Sample 1/1 in Microplate

The procedure has a linear range of 25-1000nmol/mL (primary amine concentration)

1. Pipette 75µL of each Conjugated, Unconjugated, and Biotin Control Sample into a well in three or more replicates
   Note: Different sample sizes may be used, e.g. 125µL, as long as the WR/Sample is 1/1.
2. Add 75µL WR into each well and shake gently. Cover the plate with parafilm.
3. Incubate for at least 15min at temperature between 25-37°C.
4. Remove parafilm and measure UV absorbance at 350nm in a plate reader.
5. Subtract the average absorbance of Biotin Control from that of Conjugated and Unconjugated sample replicates to obtain the corresponding corrected absorbance (CA).
6. Use the formula to calculate the Conjugation Ratio.

   Conjugation Ratio = (Unconjugated's CA - Conjugated's CA) ÷ Unconjugated's CA) x (# of Lys + # of N-Termini)

   Where
   Conjugation Ratio is the number of molecules (biotin in the case) attached to one protein (antibody);
   Conjugated's CA is the corrected absorbance of Conjugated Sample;
   Unconjugated's CA is the corrected absorbance of Unconjugated Sample;
   # of Lys is the number of un-blocked lysine in the protein before conjugation;
   # of N-termini is the number of un-blocked N-termini in the protein before conjugation.

4 Compatibility

The X-Lyz™ Conjugation Density Assay is compatible with reducing agents, common detergents, alcohols, urea, guanidine, metal chelators, and inorganic salts.

Primary amine-containing substances, such as amino acids, ammonia, and TRIS buffer interfere with the assay, is not suitable for the method and should be avoided.

Non-primary amine buffers may be used to replace TRIS, such as HEPES, BICINE, BIS-TRIS, BIS-TRIS propane, and BIS-TRIS methane.

Irvine Chemistry Lab
3303 E. Miraloma Ave., Ste 186
Anaheim, CA 92806 USA

Tel: (714) 855-4622
Fax: (714) 276-2202

info@irvinechemistry.com
www.irvinechemistry.com

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